Remarks/Arguments:

Claims 22-36, newly present hereby, are pending.

Claims 1-21 are canceled, without prejudice or disclaimer, with claims 11, 12, 18, 19, and 21 being canceled pursuant to restriction.

Newly presented claims 22-36 correspond to the elected subject matter—pursuant to the restriction requirement of record—provided in the original claims. More precisely, present claim 22 contains subject matter of original claim 8 (i.e., SEQ I D NO: 86) and original claim 2 (i.e., biological activity against infection by HIV) incorporated into original claim 1. New claims 23-25 contain subject matter from claims 6, 7, and 9, respectively. Present claims 26, 27, and 28 contain subject matter from original claim 10. Present claims 29 and 33-36 contain subject matter of original claims 13, 15-17, and 20, respectively. Present claims 30, 31, and 32 contain subject matter of original claim 14. Additionally, the present claims include formality changes—with respect to the original claims—in order to more clearly define the instant invention.

The requirements for restriction/election, of record, are repeated and made final, in the instant Office Action. Applicants maintain their traversal of the restriction/election requirements, although the present claims are limited to the elected subject matter.

Original claims 1-10, 13-17 and 20 were rejected under 35 USC 101 for allegedly being directed to non-statutory subject matter. Reconsideration is requested in view of the changes to the claims effected by the instant amendment.

According to the statement of rejection, the "peptides" originally claimed encompassed non-statutory products of nature. All of the present, pending claims are directed to—or contain—a "synthetic compound," which does not encompass a product of nature.

Accordingly, the rejection under §101 is overcome, i.e., in view of the new claims presented, hereby. Withdrawal of the rejection appears to be in order.

Claims 1-13 were rejected under 35 USC 112, first paragraph as allegedly unsupported by a sufficient written description of the invention, i.e., the instant specification allegedly failing to reasonably convey to one of ordinary skilled in the art that applicants had possession of the claimed invention at the time the application was filed. Claims 1-10, 13-17 and 20 were rejected under 35 USC 112, first paragraph, as allegedly lacking enablement. Reconsideration of the rejections under §112, ¶1, is requested, in view of the changes to the claims effected, hereby, taken in conjunction with the following remarks.

As indicated in the present Office Action (sentenced bridging pages 3 and 4), the "written description rejection—§112, ¶1—is not "an enablement rejection"—under §112, ¶1. Nevertheless, in the present case the written description is related to the enablement rejection and, therefore, the rejections are discussed, together, in the present remarks. The description requirement of §112 may be review separately from the enablement requirement, but the requirements are "intertwined." *Kennecott Corp. V. Kyocera International Inc.*, 5 USPQ2d 1194, 1197 (Fed. Cir. 1987), *cert. denied*, 486 U.S.1008 (1988).

The presently claimed subject matter is limited to a 'compound' that is

> a peptide having amino acid sequence $(LEAIPCSIPPEFLFGKPFVF)_2$ (SEQ ID NO: 86), a conservatively substituted variant of the peptide containing a maximum of two cysteine residues, or an amidated, alkylated, acylated, sulfated, pegylated, phosphorylated, or glycosylated derivative of the peptide,

a "medicament" containing the compound, and a medical-treatment "use" of the compound.

On the alleged lack of a written description, applicants dispute the allegation that they were not in possession of the recited genera (1) nucleic acid coding the peptide having SEQ ID NO: 86, i.e., $(LEAIPCSIPPEFLFGKPFVF)_2$, and (2) antibody binding specifically to the peptide. When the skilled person has knowledge of the sequence of a protein or peptide—as provided by the subject application—he also knows, implicitly, the sequence of the nucleic acid coding for such protein or peptide.

The patent law recognizes that it makes little sense to extend the written description of a patent by explaining—in the present case—what amounts to a dogma of molecular biology, i.e., a triplet of nucleic acids is responsible for the coding of each amino acid in a given protein. Since the skilled artisan is well aware of what is already known in the art, providing the same information in a patent specification would be redundant; and, therefore, a "patent need not disclose, and preferably omits, that which is well known in the art." *Hybritech, Inc. v. Monoclonal Antibodies, Inc.*, 231 USPQ 81, 94 (Fed. Cir. 1986). *Staehelin v. Secher*, 24 USPQ2d 1513, 1516 (BPA&I 1992) ("an application need not teach, and preferably omits, that which is well known in the art").

Accordingly, disclosing the sequence—$(LEAIPCSIPPEFLFGKPFVF)_2$—of the presently claimed compound/peptide implicitly discloses the nucleic acid coding for the compound recited in

the present claims. The same is true with respect to the recited antibody binding specifically to the peptide.

Decades have passed since the antibody-related technologies of Köhler and Milstein (e.g., "Continuous cultures of fused cells secreting antibody of predetermined specificity," *Nature, 256*, 1975, 495–497) were developed. It seems unnecessary to teach the ordinary skilled person—in written form in a patent application—how to make an antibody. Moreover, the presently claimed invention involves the antibodies being only a useful diagnostic tool with respect to the presently claimed compound/peptide subject matter. It should also be pointed out that any need to screen a protein—such as the presently recited antibody—for biological activity is routine, not undue, experimentation, which is consistent with the requirements of §112, ¶1. *Ex parte Mark*, 12 USPQ2d 1904 (Bd. Pat. App. & Inter. 1989).

With respect to description and enablement of using the presently claimed compound submitted herewith is the published periodical article *Cell, 129* (2007) 263-275 (Münch et al.), which deals with using peptides for the treatment of HIV.

In view of the teachings of invention provided by the subject application, given the knowledge in the prior art—as evidenced, e.g., by Münch et al.—the skilled person would have (1) considered that applicants had possession of the *generic* invention as presently claimed and (2) been enabled to practice the *generic* invention as presently claimed. Accordingly, both the written description and enablement requirements under §112, ¶1, are satisfied with respect to the presently claimed invention.

For the foregoing reasons the rejection of claims 1-10, 13-17, and 20 under §112, ¶1, for allegedly lacking enablement and the rejection of claims 1-10 and 13 and under §112, ¶1, for allegedly lacking a written description are overcome. Withdrawal of the rejections appears to be in order.

Claim 17 was rejected under 35 USC 112, 2nd ¶, for allegedly being indefinite. Reconsideration is requested in view of the changes to the claims effected by the instant amendment.

According to the statement of rejection claim 17 is indefinite for failing to recite any positive method/process steps. Present claim 35—replacing claim 17—recites the positive step "administering the compound [of claim 22] to a patient in need thereof."

Since the rejected claim is amended hereby—as claim 35—to recite a positive method/process step, the rejection under §112, ¶2, is overcome. Withdrawal of the rejection appears to be in order.

The objection to the claims is overcome by limiting the present claims to the elected peptide $(LEAIPCSIPPEFLFGKPFVF)_2$—its sugar-modified derivative and conservatively substituted variant—subject matter. Withdrawal of the objection appears to be in order.

Attorney Docket No. P70650US0
Application No. 10/539,627

Favorable action is requested.

Respectfully submitted,

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Discovery and Optimization of a Natural HIV-1 Entry Inhibitor Targeting the gp41 Fusion Peptide

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SUMMARY

A variety of molecules in human blood have been implicated in the inhibition of HIV-1. However, it remained elusive which circulating natural compounds are most effective in controlling viral replication in vivo. To identify natural HIV-1 inhibitors we screened a comprehensive peptide library generated from human hemofiltrate. The most potent fraction contained a 20-residue peptide, designated VIRUS-INHIBITORY PEPTIDE (VIRIP), corresponding to the C-proximal region of α1-antitrypsin, the most abundant circulating serine protease inhibitor. We found that VIRIP inhibits a wide variety of HIV-1 strains including those resistant to current antiretroviral drugs. Further analysis demonstrated that VIRIP blocks HIV-1 entry by interacting with the gp41 fusion peptide and showed that a few amino acid changes increase its antiretroviral potency by two orders of magnitude. Thus, as a highly specific natural inhibitor of the HIV-1 gp41 fusion peptide, VIRIP may lead to the development of another class of antiretroviral drugs.

INTRODUCTION

A variety of components in human blood and tissues have been implicated in the inhibition of HIV-1 replication in infected individuals. For example, the discovery that chemokines bind to cofactors of HIV-1 entry and block viral replication represented a major breakthrough in AIDS research and led to the development of several novel types of antiretrovirals targeting various steps in the viral entry process (reviewed in Moore and Stevenson, 2000; Ray and Doms, 2006). However, it was frequently proven difficult to purify and characterize the antiviral compound(s) released from human cells or tissues. For example, it seems that the antiviral factor released by $CD8^+$ T cells, initially described 20 years ago (Walker et al., 1986), has not yet been identified, although a variety of natural anti-HIV factors has since been detected in the long and intensive search (reviewed in Levy, 2003). Major obstacles in identifying antiviral factors circulating in the human body in a systematic, effective, and unbiased manner are the limited quantities of available human material and the lack of standardized methods to purify biologically active natural HIV inhibitors.

Progress in the generation and analysis of peptide libraries derived from human body fluids or tissues allowed these limitations to be overcome. For example, technologies have been developed for separating peptides from large amounts of up to 10,000 liter hemofiltrate (HF), which is available in abundance from patients with chronic renal failure (Forssmann et al., 1992; Schulz-Knappe et al., 1996). HF-derived peptide libraries contain an estimated number of more than one million different peptides and small proteins, representing essentially all circulating compounds with a molecular weight below 30 kDa. These include chemokines, defensins, cytokines, and hormones, in a highly concentrated and bioactive form (Schulz-Knappe et al., 1997). Previous studies demonstrated

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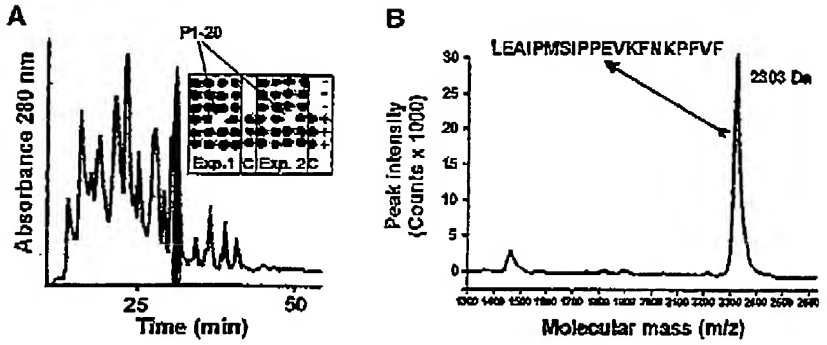

Figure 1. Purification of VIRIP from Human Hemofiltrate

(A) Hemofiltrate pH pool 1 was subjected to RP-HPLC and the resulting fractions were assayed for antiviral activity. The red area specifies the active fraction. The inlet shows inhibition of HIV-1 replication by fraction P1-20 (indicated by the arrows). Human PBMCs were infected with HIV-1 NL4-3 in the presence of peptide fractions obtained from pH pool 1. Viral replication was measured by RT assay with cell-free culture supernatant obtained at 9 days post-infection. The results of two infections are shown (Exp. 1 and 2). The symbols specify wells with uninfected cells (–) or without peptide added (+) in the two control (C) columns.

(B) MALDI-TOF analysis of the inhibitory fraction obtained at the last purification step revealed a major compound with a molecular mass of 2303 Dalton. Identification of the 20 aa sequence was conducted by Edman degradation.

that such peptide libraries represent a useful source for identification and purification of new natural anti-HIV-1 agents (Detheux et al., 2000; Münch et al., 2002). Notably, the relative concentrations of the compounds correspond to those of human plasma (Schulz-Knappe et al., 1996). Thus, these libraries should allow identification of those small circulating agents that are most relevant for controlling viral infection in vivo, at least in the absence of a specific antiviral host immune response.

Here, we performed a systematic screening of an HF-derived peptide library for novel HIV inhibitors. We identified a natural 20-residue C-proximal subfragment of α1-antitrypsin (α1-AT), designated VIRUS-INHIBITORY PEPTIDE (VIRIP), as a broad-based inhibitor of HIV-1. Moreover, we found that a few amino acid changes enhanced antiretroviral potency of VIRIP by two orders of magnitude. Functional and structural analyses revealed that VIRIP inhibits HIV-1 entry by specific binding to the gp41 fusion peptide (FP). In agreement with this distinct inhibitory mechanism VIRIP and its derivatives remained fully active even against HIV-1 strains resistant to currently available antiretroviral drugs including fusion inhibitors. Our data support the possibility that VIRIP may contribute to controlling HIV-1 replication in infected individuals and that derivates thereof are highly suitable for development of a new class of HIV-1 inhibitors targeting the highly conserved gp41 FP.

RESULTS

Isolation of an HIV-1 Inhibitor from Human Hemofiltrate

A peptide library was established by enrichment and separation of peptides from human HF using cation exchange and reversed phase (RP) chromatography (Schulz-Knappe et al., 1997). A total of 322 different peptide-containing RP-HPLC fractions from the HF peptide library were screened to identify factors that block wild-type HIV-1 NL4-3 (NL4-3wt) replication in human PBMCs. One fraction (No. P1-20) was selected for detailed analysis because it was not cytotoxic and displayed potent anti-HIV-1 activity, indicated by the lack of detectable reverse transcriptase (RT) activity in the culture supernatant (Figure 1A). The active peak was further purified by two additional chromatographic steps. MALDI-TOF analysis of the inhibitory fraction obtained after the final step revealed a major peak with a molecular mass of 2303 Da (Figure 1B). Edman degradation identified the sequence LEAIPMSIPPEVKFNKPFVF showing that this peptide, VIRIP, corresponds exactly to residues 353–372 of human α1-AT (accession number P01009), the most abundant circulating serine protease inhibitor (Travis and Salvesen, 1983). The α1-AT is produced mainly by the liver and controls proteinases in many biological pathways even though its main function is to protect the lung against proteolytic damage from neutrophil elastase (Stoller and Aboussouan, 2005).

VIRIP Inhibits HIV-1 Infection and Transmission

To exclude the possibility that a contaminating agent might be responsible for the observed effects, we next tested chemically synthesized VIRIP for antiviral activity. The synthetic peptide inhibited infection of PBMC by X4- and R5-tropic HIV-1 with a mean 50% inhibitory concentration (IC_{50}) of about 20 μM but had no effect on infection by viral particles pseudotyped with vesicular stomatitis virus (VSV-G) or murine leukemia virus (MLV) Env glycoproteins (Figure 2A). Importantly, VIRIP was not cytotoxic

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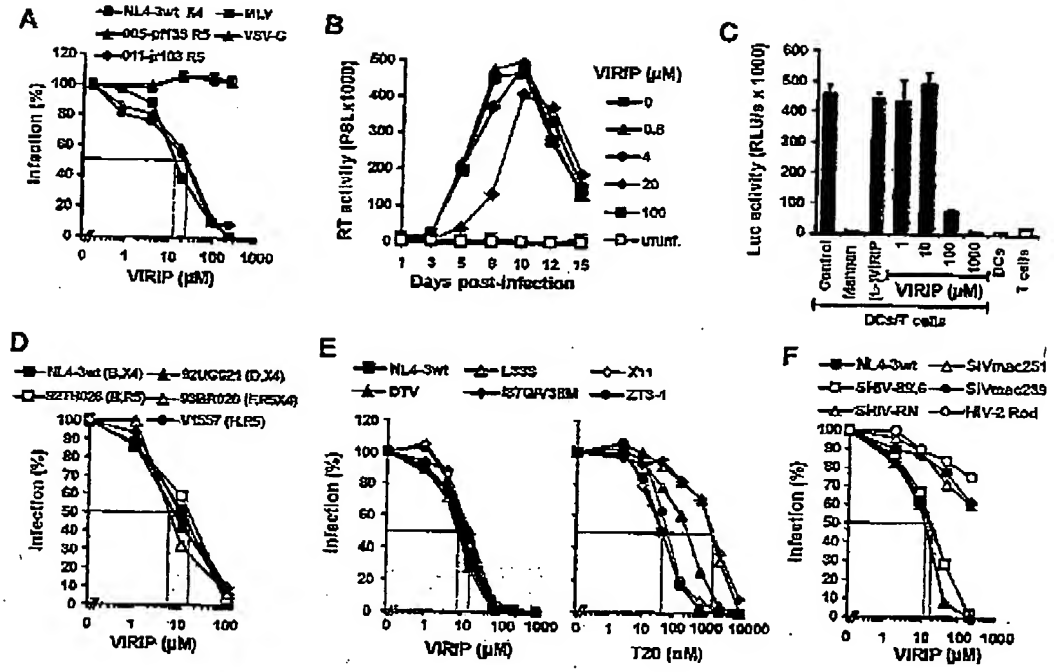

Figure 2. Synthetic VIRIP Inhibits HIV-1 Replication and Transmission

(A) PBMCs were infected with X4-tropic NL4-3wt and R5-tropic V3 recombinants (blue) or with HIV-1 particles carrying heterologous VSV-G or MLV Env proteins (green) all containing the *luciferase* reporter gene instead of *nef* in the presence of the indicated concentrations of VIRIP. Results shown in all panels were confirmed in two to five independent experiments.

(B) Prestimulated PBMCs were infected with HIV-1 NL4-3 in the absence or presence of an inhibitor. Supernatants were harvested at regular intervals and analyzed for RT activity. PSL, photo-stimulated luminescence.

(C) DCs were exposed to equal amounts of HIV-1 NL4-3 encoding the *luciferase* gene, washed, and cocultivated with autologous primary CD4$^+$ T cells in the presence of the indicated concentrations of VIRIP. As controls, DCs or T cells alone were infected with the same viral dose. RLU/s, relative light units per second. Shown are averages values (±SD) derived from three infections.

(D–F) Dose-dependent inhibition of (D) primary HIV-1 isolates of different subtype and coreceptor usage (indicated in parentheses); (E) HIV-1 NL4-3 variants resistant to protease as well as nucleoside and non-nucleoside RT inhibitors (X11 and ZT3-1) and the fusion inhibitor T20 (DTV: L33S, I37Q/V38M); and (F) HIV-1, HIV-2, and SIVmac and chimeric SIVmac239 clones containing HIV-1 89.6 and RN Env proteins. All curves give average values obtained from triplicate infections.

even at exceedingly high concentrations (Figure S1). HIV-1 NL4-3 replication in PBMCs was suppressed at 20 μM and completely blocked at 100 μM (Figure 2B). Further experiments confirmed that both isolated and chemically synthesized VIRIP inhibit HIV-1 infection with the same potency (data not shown). Binding of HIV-1 to DC-SIGN on dendritic cells (DCs) in the periphery, and subsequent transport and transmission to T cells in secondary lymphoid organs, may be important for the spread of HIV-1 in humans (Geijtenbeek et al., 2000). We found that VIRIP blocked HIV-1 transmission by monocyte-derived DCs to T cells at 100 μM (Figure 2C). Thus, even the presence of an efficient virus attachment factor did not overcome the ability of VIRIP to inhibit viral infection. We next examined the effect of VIRIP on HIV-1 infection of P4-CCR5 indicator cells (Charneau et al., 1994) expressing CD4 and both major entry cofactors CXCR4 and CCR5. VIRIP inhibited primary HIV-1 group B subtype A, B, C, D, F, and H strains and several group O isolates with IC_{50} values ranging from 4 to 20 μM (examples shown in Figure 2D). Thus, the inhibitory activity of VIRIP was independent of viral subtype or coreceptor usage. Notably, VIRIP also blocked HIV-1 variants resistant to protease and RT inhibitors (X11, ZT3-1) (Walter et al., 2000) and the fusion inhibitor T20 (DTV, L33S, I37Q/V38M) (Armand-Ugon et al., 2003; Rimsky et al., 1998; Figure 2E), suggesting that it may be effective in patients failing current HAART regimens. In comparison to all HIV-1 variants analyzed, the HIV-2 Rod, SIVmac239, and SIVmac251 strains were hardly susceptible to VIRIP inhibition (Figure 2F). However, infection by SIV particles carrying HIV-1 Env proteins (SHIV-89.6 and SHIV-RN) (Miyazaki et al., 2002; Reimann et al., 1996) was blocked (Figure 2F). Thus, our data show that VIRIP is a broad-spectrum inhibitor of HIV-1 Env function.

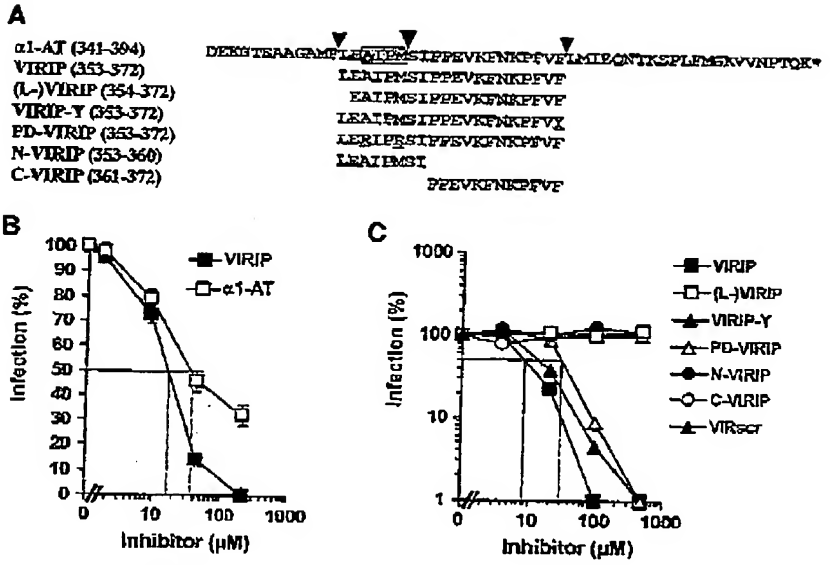

Figure 3. Antiviral Effect of Full-Length α1-AT and VIRIP-Related Peptides

(A) C-terminal amino acid sequence of α1-AT and peptides analyzed. The protease cleavage site in the reactive center loop and the cleavage sites for VIRIP are indicated by red and blue triangles, respectively. The four residues belonging to the reactive center of α1-AT are highlighted in yellow, the asterisk indicates a stop codon, and the amino acids in the peptides differing from those found in α1-AT are underlined.

(B and C) Infection of P4-CCR5 cells with HIV-1 NL4-3 in the presence of (B) full-length α1-AT or (C) a variety of C-terminal fragments of α1-AT and a scrambled form of VIRIP. A dotted line indicates 50% inhibition. None of the compounds showed cytotoxic effects at the concentrations used in the inhibition studies. Shown are average values derived from triplicate infections.

Specificity of VIRIP

To further evaluate the specificity of VIRIP, we tested the antiviral effects of full-length α1-AT and several VIRIP-related C-terminal fragments of α1-AT (Figure 3A). As expected from a previous study (Shapiro et al., 2001), α1-AT also inhibited HIV-1 infection. However, the full-length molecule was less potent than VIRIP, blocking HIV-1 infection by a maximum of 70%, whereas equimolar concentrations of VIRIP caused 99% inhibition (Figure 3B). We found that after 24 to 48 hr incubation in human plasma, about 50% of VIRIP was converted into an N-terminally truncated derivative, (L-)VIRIP, lacking the first leucine (data not shown). Surprisingly, this single amino acid deletion resulted in a complete loss of antiviral activity (Figure 3C). We also investigated the effect of substitutions of A355R and M358R (numbering corresponding to the full-length α1-AT sequence). These alterations are present in the α1-AT Portland variant, a high-affinity inhibitor of furin (Anderson et al., 1993), one of the proteases cleaving the gp160 Env precursor (Hallenberger et al., 1992). However, introduction of these changes into VIRIP (PD-VIRIP) led to a decrease in its antiviral potency. Further analysis confirmed that VIRIP does not inhibit serine proteases (data not shown). In addition, the N- and C-terminal moieties and a scrambled form of VIRIP (VIRscr) were also inactive (Figure 3C). Thus, the 20-residue-comprising VIRIP sequence blocks HIV-1 infection in a highly specific manner.

Improved Antiviral Potency

To enhance the antiviral activity of VIRIP, we performed a systematic structure-activity relationship (SAR) analysis. At first, all possible positions in the original peptide were replaced by L-alanine to determine critical residues. Analysis of these VIRIP derivatives revealed that the side chains at the four N-terminal (LEAI) and the three C-terminal (FVF) residues are crucial for antiviral activity, whereas the remaining alterations were tolerated (Table S1). Notably, the K13A substitution resulted in a peptide with significantly increased antiviral potency and reduced net charge. Furthermore, we examined VIRIP derivatives with an N-terminal acetyl group or with amidated C terminus. The results demonstrated that the negative N-terminal charge is crucial for antiviral activity (Table S1). In addition, we found by testing peptides of a D-amino acid scan that an epimer containing D-proline at position 10 also showed increased antiviral potency.

To carry out advanced SAR studies, further series of >600 VIRIP analogs were designed and chemically synthesized, among them derivatives that contained a variety of natural and non-natural amino acids at positions, which tolerated substitution by alanine (examples shown in

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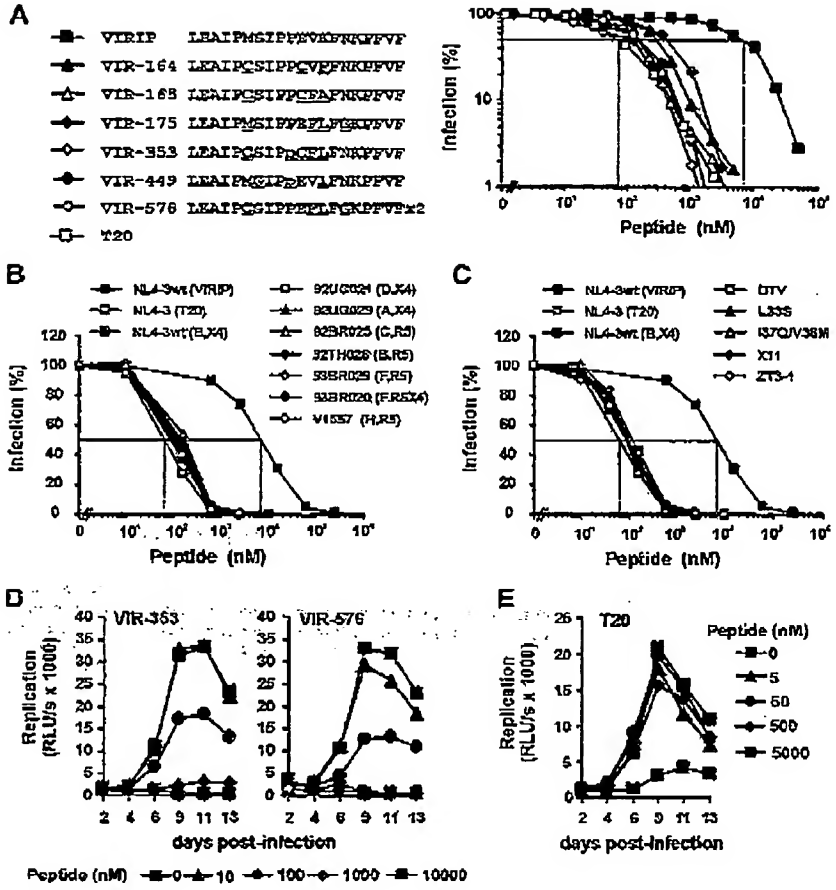

Figure 4. Improved Antiviral Potency of VIRIP Derivatives

(A) P4-CCR5 indicator cells were infected with NL4-3 stocks containing 1.0 ng p24 antigen in the presence of VIRIP, a variety of VIRIP derivatives (amino acid sequence shown in left panel; p, D-proline; cysteine residues are linked via a disulfide bridge), or T20. Average values obtained from quintuple infections are shown (right panel). The results were confirmed in two to five independent experiments.

(B and C) Dose-dependent inhibition of (B) primary HIV-1 isolates of different subtype and coreceptor usage (indicated in parentheses) or (C) RT-, protease-, and T20-resistant HIV-1 variants by VIR-576. NL4-3wt inhibition by VIRIP and T20 is shown for comparison. All curves give average values obtained from triplicate infections. Similar results were obtained in two independent experiments.

(D and E) Inhibition of R5-tropic HIV-1 replication in ex vivo infected human tonsillary lymphocyte aggregate cultures by (D) VIR-353 and VIR-576 and (E) T20. Shown are average luciferase activities in culture supernatants derived from triplicate infections. Similar results were obtained with another R5-tropic HIV-1 clone and X4-tropic NL4-3wt. RLU/s, relative light units per second.

Table S1). An additional approach to increasing antiviral potency was the introduction of an intramolecular cyclic motif by a disulfide bond. For this, residues were replaced by cysteines to generate disulfide bonds. Such peptides are considered to exhibit a more rigid structure with less flexibility. Several of these VIRIP derivatives displayed about two orders of magnitude increased anti-HIV-1 activity compared to the natural form (examples shown in Figure 4A). Thus, some of them were equally potent as T20 in blocking NL4-3wt infection (Figure 4A). The modified forms of VIRIP were also highly active against diverse primary HIV-1 strains and against RT-, protease-, and fusion inhibitor-resistant virus variants (VIR-576 is shown as an example in Figures 4B and 4C). Examination of the infected cell cultures by electron microscopy confirmed that VIR-576 protected the cells against cytopathic effects and prevented virus production (Figure S2). Moreover, VIRIP derivatives were highly effective in blocking trans HIV-1 transmission from DCs to primary T cells (Figure S3) and maintained significant antiviral activity

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even after several days of incubation in human serum (VIR-576 shown as an example in Figure S4). Thus, the antiviral potency of VIRIP can be greatly improved by changing specific amino acid side chains and/or particular structural features such as charge and peptide chain rigidity.

To further assess the antiviral potency of VIRIP derivatives we infected human lymphocyte aggregate cultures (HLAC) of tonsils (Eckstein et al., 2001) with HIV-1 ex vivo in the presence or absence of an inhibitor. Lymphoid tissue is the site where most HIV-1 replication and the critical events in the progression of AIDS occur in vivo (reviewed in Stevenson, 2003). Thus, this ex vivo organ culture system provides a relevant model for studying the inhibitory effect of antiviral agents on HIV-1 replication in humans. We found that VIR-353 and VIR-576 as investigated model analogs markedly inhibited the spread of HIV-1 in ex vivo infected HLAC cultures at 100 nM and completely blocked viral replication at 1 μM (Figure 4D). In comparison, T20 suppressed HIV-1 replication at 5 μM but not at 500 nM (Figure 4E). Thus, VIRIP derivatives are potent inhibitors of HIV-1 replication in HLAC. Further studies confirmed that VIRIP derivatives are active against a wide variety of HIV-1 strains without causing cytotoxic effects (Figure S1 and data not shown). In summary, our findings show that VIRIP derivatives are highly promising for further clinical development.

Inhibitory Mechanism of VIRIP

Our results showed that VIRIP blocks specifically the function of the HIV-1 Env protein (Figure 2A). In agreement with these data, the original peptide inhibited HIV-1 Env-mediated cell fusion with an IC_{50} of 26 μM (Figure 5A). Optimized derivatives were about 100-fold more effective than VIRIP, whereas (L-)VIRIP or VIRscr (Table S1) were inactive. We next investigated which step of the HIV-1 entry process is blocked. As shown in Figure S5, VIRIP and its derivatives did not alter CD4 and CCR5 or CXCR4 coreceptor expression. In contrast to anti-CD4 antibodies and the small molecule CCR5 inhibitor TAK-779, they also did not affect binding of HIV-1 gp120 to CD4 or CCR5 (Figure S6). Next, we examined whether VIRIP derivatives inhibit six-helix bundle (6-HB) formation by the gp41 N- and C-proximal heptad repeat (NHR and CHR, respectively) regions, as do other HIV-1 fusion inhibitors (Kilby et al., 1998; Root et al., 2001). This interaction can be mimicked with synthetic N and C peptides corresponding to the NHR and CHR regions, respectively, (Lu et al., 1995) and detected by ELISA (Gallo et al., 2006; Jiang et al., 1999) using a monoclonal antibody (NC-1) binding specifically to the complex but not to the individual peptides (Jiang et al., 1998). Unlike the fusion inhibitors T1249 and C34 (Armand-Ugon et al., 2003; Eron et al., 2004), none of the VIRIP derivatives inhibited 6-HB formation (Figure 5B). As expected from published data (Liu et al., 2005), T20, which does not contain the cavity-binding domain (Chan et al., 1998), did not efficiently inhibit 6-HB formation of the fusogenic core.

Altogether, our results strongly suggested that VIRIP does not affect CD4 binding, coreceptor interaction, or formation of 6-HB. Thus, we next studied whether VIRIP might interfere with the function of HIV-1 gp41 FP (FP1-23). Incubation of human erythrocytes with synthetic highly hydrophobic FP usually results in significant hemolysis (Mobley et al., 1992). We found that the hemolytic activity of FP1-23 was clearly inhibited by VIRIP but not by (L-)VIRIP or VIRscr (Figure 5C). Notably, VIRIP derivatives showing improved anti-HIV-1 activity (Figure 5D) were also more potent in preventing HIV-1 gp41 FP-induced hemolysis (Figures 5C and S7). Optimized VIRIP derivatives also inhibited the hemolytic activity of the SIV/HIV-2 FP, albeit only at higher concentrations. However, they did not affect hemolysis induced by MLV FP (Figure 5C). We found that T20 also displayed antihemolytic activity. This was not surprising because T20 contains a high ratio of hydrophobic residues and has been previously shown to reduce HIV-1 gp41 FP-induced hemolysis (Mobley et al., 2001). However, the effect on the HIV-1 and SIV/HIV-2 FPs was weaker than that on the MLV FP (Figure 5C), although the latter virus is not susceptible to T20 inhibition. These data suggest that T20 inhibits the hemolytic activity of different FPs largely via nonspecific hydrophobic interactions. In contrast, the antihemolytic activity of VIRIP and its derivatives was highly specific and correlated with their antiviral potency.

To assess whether alterations in the FP might affect the sensitivity of HIV-1 to VIRIP inhibition, we replaced the FP of NL4-3 with that of SIVmac239 (which is identical to that of HIV-2). We found that significantly higher concentrations of VIRIP and its derivatives (average of 4.6 ± 1.4-fold; n = 8) were required for 50% inhibition of the FP mutant compared to the parental HIV-1 clone (Figure 5D and Table S2). In contrast, viral sensitivity to T20 inhibition was 5.6-fold enhanced by exchanging the FP. We also examined SIVmac239 containing the HIV-1 FP but this chimera was not infectious (data not shown). SIVmac239 was substantially less sensitive to VIRIP inhibition than the HIV-1 chimera containing the SIV/HIV-2 FP, although up to 70% inhibition of SIV was observed in the presence of relatively high concentrations of optimized VIRIP derivatives (Figures 5C and S8). Taken together, the results of the hemolysis and inhibitions studies show that VIRIP derivatives interact strongly with the HIV-1 FP, less efficiently with the SIV/HIV-2 FP, and not at all with the MLV FP. However, possibly due to altered fusion kinetics and/or accessibility of the FP, the viral envelope backbone also affects the susceptibility of HIV-1 to VIRIP inhibition.

Structure of the Complex between VIR-165 and the gp41 FP

To further elucidate the inhibitory mechanism we analyzed the binding specificity of the optimized VIR-165 derivative (Figure 4A) with gp41 FP1-23 by NMR spectroscopy. To facilitate the NMR assignments the measurements were performed at pH 4.7, which is lower than under physiological conditions (pH 7.2). The fact that the two peptides are

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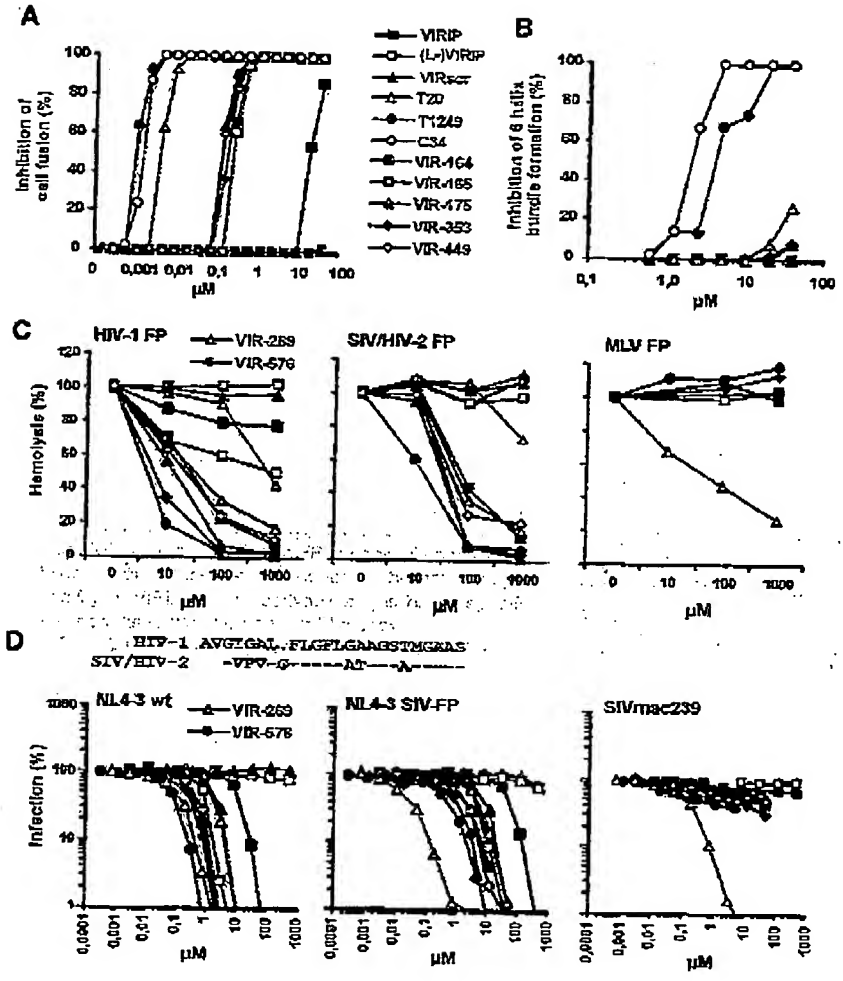

Figure 5. Inhibitory Mechanism of VIRIP

(A) Inhibition of HIV-1 Env-mediated cell fusion by the indicated peptides. Identical symbols are also used in (B)–(D). Average values of triplicate measurements are shown in (A)–(C).

(B) Inhibitory effect of VIRIP derivatives and fusion inhibitors on gp41 6-HB formation.

(C) Inhibitory effect of VIRIP derivatives and T20 on hemolysis induced by HIV-1, SIV/HIV-2, and MLV FPs.

(D) Inhibition of NL4-3wt, an NL4-3 chimera containing the SIV/HIV-2 FP and SIVmac239, by various VIRIP derivatives and T20. The upper panel shows an alignment of the gp41 FP sequences of HIV-1 and SIV/HIV-2. Dashes and the dot indicate amino acid identity and a gap, respectively. Average values obtained from sextuple infections of P4-CCR5 indicator cells are shown.

interacting was confirmed by NMR spectroscopy using nuclear Overhauser and exchange spectroscopy (NOESY) spectra at 250 MHz. At this frequency the NOEs of the free peptides are still positive, indicative of the monomeric nature of the peptides. However, the NOESY spectrum of a mixture of the two peptides at 250 MHz shows negative signs of the NOEs proving the formation of a heterodimeric complex in solution.

To quantify the interaction of VIRIP derivatives with the gp41 FP we performed Biacore studies. Results obtained using immobilized HIV-1 gp41 FP and prototype VIRIP derivatives showed that VIR-165 binds about 20-fold more strongly to FP1-23 than (L-)VIRIP (data not shown). This leads to a 60-fold faster association as evident from the association rates (k_{on}) of VIR-165 (k_{on} = 3.2 × 10^5 $M^{-1}s^{-1}$) and (L-)VIRIP (k_{on} = 5.4 × 10^3 $M^{-1}s^{-1}$). These

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data suggest that (L-)VIRIP is not assuming a structure as well defined as that of VIR-165, indicating that the first leucine in VIRIP is critical for antiviral activity because it stabilizes the secondary structure of the N-terminal section. The analysis of the binding kinetics from VIR-576 yielded $k_{on} = 4.5 \times 10^5\ M^{-1}s^{-1}$, whereas VIRscr showed no specific binding. Thus, only the two inhibitory prototype VIRIP derivates but not the inactive control peptides associated efficiently with the HIV-1 gp41 FP.

Combination of NMR data obtained at 700 MHz and at 500 MHz proved to be beneficial for the complete assignment of all NH and CHα resonances of FP1-23 and VIR-165 (summarized in Tables S3 and S4). An almost complete assignment of side-chain protons was possible. The conformation of the individual peptides was obtained from NOESY spectra in buffered solution. Long-range interactions with the associated distances are listed in Table S5. The structure of the complex of FP1-23 and VIR-165 was further analyzed by NOESY experiments applied to an equimolar mixture of the two peptides (not shown). Unfortunately, it was difficult to unambiguously assign many of the intermolecular NOESY crosspeaks even at 700 MHz since the chemical shifts of the protons of the two peptides are very similar, thus resulting in a considerable overlap of the signals obtained in the NOESY spectra of the mixture. On the basis of the NH, CHα, and side-chain assignments, key intermolecular NOE crosspeaks were identified (Table S6). Importantly, these show the close proximity of the newly introduced residue F12 of VIR-165 to L12 of FP1-23.

Using unambiguous key intermolecular NOEs between the two peptides in the complex, it was possible to construct a docking model of FP1-23 and VIR-165. The conformation of the two peptides was initially kept the same as determined in solution. Applying the key intermolecular NOEs the DYANA distance geometry simulation yielded a complex in which both peptides basically remained in the respective structure that they had each adopted separately in the solution. This model was then used as a starting model for a refinement procedure utilizing an MD simulation with the TRIPOS force field. During this 500 ps simulation all NOE restraints, including intermolecular ones, were active and yielded a final refined structure of the complex of FP1-23 and VIR-165. The resulting model is in excellent agreement with the experimental constraints as seen in Table S6. Our data also show that the two peptides already adopt their bioactive conformation before the complex is formed. Large highly complementary hydrophobic surfaces are responsible for the strong and remarkably specific interaction between VIR-165 and the gp41 FP (Figure 6). The model also explains the enhanced antiviral activity of VIR-165 as compared to VIRIP. The mutated amino acids in VIR-165, i.e., F12 and A13, have close proximity to and make hydrophobic contact to residues of FP1-23. The two newly introduced cysteine residues stabilize the active conformation of VIR-165, which is necessary for its tight interaction with FP1-23.

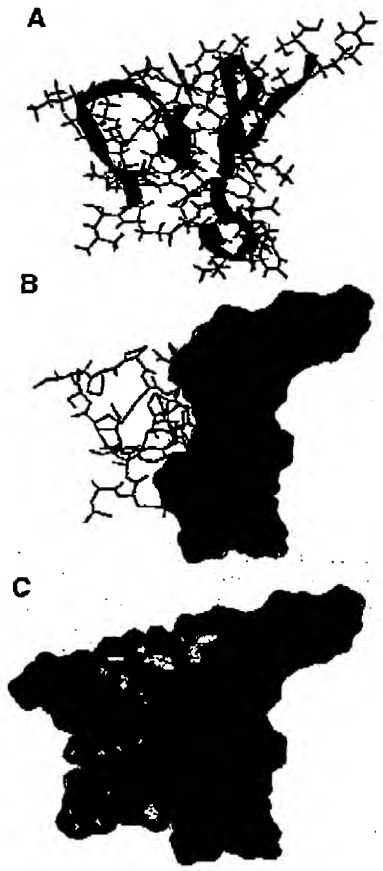

Figure 6. Structure of the VIRIP-FP Complex
(A–C) The complex of the two peptides: left VIR-165 (red and light green), right FP1-23 (blue and magenta). The amino acids interacting with each other are shown in light green and blue, respectively. The N-terminal amino acid of VIR-165, i.e., leucine, is shown in green. (A) The peptides FP1-23 and VIR-165 as ribbon and stick models; (B) peptide FP1-23 as surface plot and the inhibitor VIR-165 as stick model (hydrogen atoms not shown); (C) the two peptides VIR-165 and FP1-23 shown as surface plots. The complementarity of the surfaces is self-evident.

The HIV-1 gp41 FP Is a Useful Drug Target

To select resistant variants we initially performed serial passages of NL4-3 or mixtures of molecular HIV-1 clones in PBMCs in the presence of VIRIP, T20, or T1249, respectively. We found that no VIRIP- or T1249-resistant forms of HIV-1 emerged within two months of weekly passage. On the other hand, as expected based on previous studies (Rimsky et al., 1998), T20-resistant HIV-1 isolates containing changes in the "GIV" motif of the gp41 HR1 region emerged after 3 to 4 passages (data not shown). To clarify

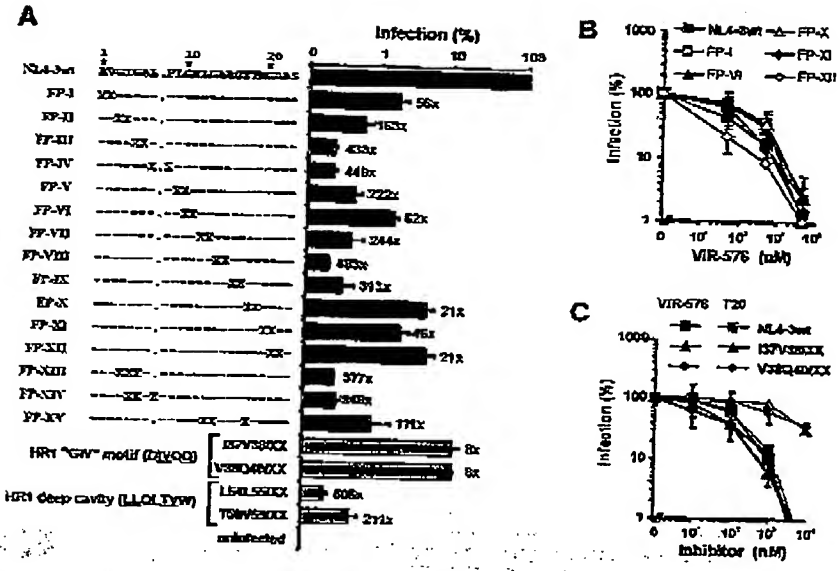

Figure 7. Mutational Analysis of the HIV-1 gp41 FP Region

(A) Infectivity of HIV-1 NL4-3 gp41 variants containing randomized codons in the HR1 region. The positions of the randomized residues in the FP region are indicated. Viral infectivity was measured by infection of TZM-bl cells with virus stocks containing normalized amounts (10 ng) of p24 core antigen and represents average values ±SD derived from triplicate infections. The numbers after the infectivity bars indicate n-fold reduction of infectivity compared to NL4-3wt virus. Similar results were obtained in three independent experiments and with CEM-M7 indicator cells. Controls are shown in green, FP mutants displaying >100-fold reduced infectivity compared to NL4-3wt in red, and those showing <100-fold reduction in infectivity in blue.

(B and C) Inhibition of HIV-1 mutants containing random changes in the (B) gp41 FP region or (C) in the "GIV" motif by VIR-576 and T20, respectively. NL4-3wt inhibition is shown for comparison. The infectivity of the remaining FP variants and of the two HIV-1 mutants containing changes in the deep cavity region was too low for meaningful analysis. All curves give average values ±SD obtained from triplicate infections.

why it might be difficult to select VIRIP-resistant virus variants, we generated a set of 15 HIV-1 mutants, containing random changes in the gp41 FP region (Figure 7A). As controls, we randomized residues in or near the "GIV" motif (known to play a key role in T20 resistance; reviewed in Greenberg and Cammack, 2004) and in the deep hydrophobic cavity (known to be highly conserved and to represent a good drug target; Chan et al., 1998) of the NL4-3 gp41 HR1 region. All proviral plasmid preparations represented a large number of independent transformants (984 ± 361; average ± SD, n = 19) and contained a complex mixture of nucleotide changes in the targeted codons (Figure S9 and data not shown). HIV-1 mutants containing random changes in the FP or deep cavity regions usually showed grossly impaired infectivity, whereas changes in the "GIV" motif were better tolerated (Figure 7A). We found that none of the FP mutant virus stocks displayed significantly reduced sensitivity to VIR-576 (Figure 7B). In contrast, the two HIV-1 pools containing changes in the gp41 "GIV" motif were resistant to T20 but not to VIR-576 (Figure 7C). No resistant virus variants were detected after propagation of the 15 HIV-1 mutants containing random changes in the gp41 FP region in CEM-M7 cells in the presence of VIR-576, whereas analogous experiments allowed the effective selection of T20-resistant forms (data not shown). These results show that changes in the gp41 FP region usually either are not tolerated or do not confer resistance to VIR-576, which likely explains the difficulty to generate VIRIP-resistant HIV-1 variants in vitro.

DISCUSSION

An Inhibitor Targeting the HIV-1 gp41 FP

This study demonstrates that a 20-residue C-proximal fragment of α1-AT is a potent broad-spectrum inhibitor of HIV-1 since VIRIP blocks infection by all HIV-1 variants tested, regardless of their subtype or coreceptor usage. VIRIP specifically inhibits HIV-1 Env function and hardly, or not at all, affects infection by virions containing HIV-2, SIV, MLV, or VSV Env proteins or by other human pathogens such as Ebola, Herpes Simplex, or Respiratory Syncytial Virus (data not shown). To date the most advanced HIV-1 entry inhibitors target CD4 binding, coreceptor interaction, or gp41-mediated membrane fusion (reviewed in Jiang et al., 2002; Moore and Stevenson, 2000; Ray

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and Doms, 2006). In contrast, VIRIP and its derivatives seem to act by a distinct mechanism. Our functional and structural data consistently indicate that VIRIP derivatives inhibit HIV-1 entry by binding to the gp41 FP and preventing its insertion into the target cell membrane. In agreement with our experimental results (Figures 2E and 4C), this property implies that VIRIP derivatives are active against HIV-1 variants that are resistant to other entry inhibitors making them highly suitable for further clinical development.

The significantly reduced susceptibility of the HIV-1 chimera containing the SIV/HIV-2 FP (Figure 5D and Table S2) shows that mutations in the gp41 FP region can confer partial resistance to VIRIP inhibition. We found, however, that it is difficult to select VIRIP-resistant HIV-1 variants in vitro. This is not entirely unexpected because the gp41 FP region is highly conserved, and it has also proven challenging to obtain HIV-1 mutants resistant against 2nd generation fusion inhibitors (reviewed in Ray and Doms, 2006). Our results show that most changes in the gp41 FP region are usually not tolerated or do not confer resistance to an optimized VIRIP derivative (Figure 7). Moreover, a combination of changes (seven in the case of the chimeric virus) seems required for significant resistance. Altogether, these results explain the difficulty to select VIRIP-resistant HIV-1 variants in cell culture. The FP hybrid virus shows enhanced susceptibility to T20 inhibition (Table S2). The reason for this remains elusive, but obviously decelerated fusion kinetics of the chimeric gp41 could be responsible for this phenotype and may also explain why the HIV-1 SIV-FP chimera is more susceptible to inhibition by VIRIP derivatives than SIVmac239 (Figure 5D).

It has been previously shown that full-length α1-AT also affects HIV-1 replication (Shapiro et al., 2001). We confirmed these findings but also found, however, that VIRIP inhibits HIV-1 with higher efficacy than full-length α1-AT. Our data also imply that the modes of action are different. VIRIP does not affect HIV-1 promoter activity and, unlike α1-AT, does not inhibit HIV-2 Rod infection. VIRIP exhibits no significant inhibitory effect on a number of serine proteinases, including human neutrophil elastase, trypsin, thrombin, and tryptase and does not affect cleavage of the gp160 Env precursor into gp120 and gp41 (data not shown). Thus, our data demonstrate that VIRIP acts via a different mechanism and is a more potent inhibitor of HIV-1 than full-length α1-AT.

Possible Role of VIRIP in HIV-1-Infected Individuals

VIRIP was isolated from a complex peptide library representing essentially all low-molecular-weight compounds circulating in human blood (Foressmann et al., 1992). Fraction P1-20 was more potent in inhibiting HIV-1 than all remaining HF-derived peptide fractions, suggesting a relevant role of the inhibitory compound in vivo. The anti-HIV-1 potency of the original fraction indicates that the plasma concentration of VIRIP is about 1 μM (Figure 1A). Exact quantification of VIRIP in human plasma proved to be complicated because quantitative separation from full-length α1-AT and other inactive C-terminal subfragments was difficult to achieve. It should be noted, however, that α1-AT is the most abundant circulating serpin reaching plasma concentrations up to 250 μM during acute infection or inflammation (reviewed in Brantly et al., 1988; Kushner, 1988). Under these conditions less than 5% of the precursor molecule needs to be converted to VIRIP to reach the in vitro IC_{50}. We, as well as others, found that matrix metallo-proteinases (MMPs) 2 and 9 generate the N terminus ($F_{358} \downarrow L_{359}$) of VIRIP (Desrochers and Weiss, 1988) (data not shown). Most likely, related proteases cleave at the C terminus of VIRIP containing the same amino acid motif ($F_{376} \downarrow L_{379}$). MMPs are elevated in response to inflammation or HIV infection (Goetzl et al., 1996; Johnston et al., 2000). Accordingly, HIV-1 infection presumably induces both, the α1-AT precursor and the proteases involved in the generation of VIRIP. In summary, our results indicate that VIRIP might impact disease progression in HIV-1-infected individuals.

Complex between the HIV-1 gp41 FP and an Improved VIRIP Derivative

A complete sequential assignment of the peptides was achieved using 700 MHz NMR experiments. Long-range NOEs allowed us to elucidate the solution structures of FP1-23 and VIR-165. The analysis of the mixture of the peptides utilizing NOESY experiments revealed that FP1-23 and VIR-165 associate mainly via hydrophobic interactions of the amino acid side chains. A model of the structure generated using distance geometry (DYANA) and molecular dynamics calculations shows clearly that the high specificity of the recognition of FP1-23 by VIR-165 is due to remarkably complementary surfaces of the two peptides (Figure 6). The model also explains the enhanced specificity and affinity of VIR-165 compared to the parent VIRIP and the results of our comprehensive SAR analysis of >600 VIRIP analogs. Additional hydrophobic amino acids in VIR-165 or other optimized VIRIP derivatives increase the hydrophobic contact area between the two complementary surfaces. In contrast, changes in the C-terminal (FVF) residues of VIRIP disrupt antiviral activity (Table S1) because they eliminate critical hydrophobic interactions with residues in the gp41 FP (Figure 6). Our results further indicate that a disulfide bridge stabilizes and elimination of the first leucine destabilizes the active conformation of VIRIP required for effective gp41 FP binding and hence antiviral activity. The solution structure of the FP1-23/VIR-165 complex not only explains the antiviral activity of VIRIP and its derivatives but also provides a basis for a rational approach to design even more potent inhibitors.

Perspectives

VIRIP exhibits interesting properties for further therapeutics/drug development. It is active against a broad variety of HIV-1 variants and targets the highly conserved gp41 FP. Our studies show that the gp41 FP region is an

attractive drug target and demonstrate that it is difficult to obtain VIRIP-resistant HIV-1 variants, at least in cell culture. Furthermore, we found that many VIRIP derivatives are highly stable in human plasma and not cytotoxic even at exceedingly high concentrations. For selected VIRIP derivatives the lack of toxicity has also been confirmed in animal models. Importantly, a few amino acid changes enhance the antiviral potency of VIRIP by about two orders of magnitude. Thus, further development of VIRIP as an antiviral agent may lead to a new class of entry inhibitors expected to block HIV-1 variants resistant to currently available antiretroviral agents.

EXPERIMENTAL PROCEDURES

Identification of VIRIP

Peptides were extracted from 10,000 liters of human HF derived from patients with chronic renal disease and fractionated by cation-exchange (pH pool fractions 1–7). Subsequently, RP chromatography was performed, where 46 fractions per pool were obtained, as reported (Forssmann et al., 1992). The resulting 322 fractions were tested for their ability to block HIV-1 NL4-3 replication in PBMCs as described (Dettheux et al., 2000). Fraction P1-20 displaying strong anti-HIV-1 activity was further separated by two additional RP-HPLC steps, and biochemical characterization of the purified peptide was performed as documented (Mark et al., 1999).

Peptide Synthesis

All peptides were prepared by Fmoc solid-phase synthesis as described (Fields and Noble, 1990) and subsequently purified by reversed-phase chromatography. Identity and homogeneity of the products were analyzed by reversed-phase HPLC, capillary zone electrophoresis, electrospray MS, and sequence analysis.

HIV-1 Variants and Virus Stocks

Primary HIV-1 isolates and HIV-2 Rod were obtained through the NIH AIDS Reagent Program. Molecular clones and the generation of virus stocks are described in the Supplemental Experimental Procedures.

HIV-1 Infectivity, Replication, and Transmission

The inhibitory effect of the peptides on viral replication in PBMCs and on infectivity in P4-CCR5 cells was assayed as described previously (Dettheux et al., 2000; Münch et al., 2002). Inhibition of PBMC infection was measured by infection with NL4-3 variants containing the *luciferase* gene in place of *nef* as described (Hiebenthal-Millow and Kirchhoff, 2002). Immature DCs were generated as described (Prechtel et al., 2005). A luciferase encoding NL4-3-based reporter virus was added to 5000 immature DCs in a total volume of 140 µl. After 2 hr unbound virus was removed and cells were resuspended in 100 µl RPMI. Then, 20 µl of the various VIRIP dilutions or (L-)VIRIP (1000 µM) and 5000 PHA/IL-2-stimulated PBMC containing autologous serum (80 µl) were added to the DC cultures. As control DCs were treated with Mannan (500 µg/ml) prior to infection. Luciferase activities were detected at 3 days post-infection.

Human Tonsillary Lymphocyte Aggregate Cultures

Human lymphoid tissues were processed and cultured as described (Eckstein et al., 2001). Infections are described in the Supplemental Experimental Procedures.

Gp41 Six-Helix Bundle Formation

The conformation-specific mAb NC-1 (Jiang et al., 1998) was used in a modified ELISA as previously described (Sista et al., 2009) to determine the effect of VIRIP and its derivatives on the gp41 6-HB formation between N36 and biotinylated C34 (C34-biotin). For details, see the Supplemental Experimental Procedures.

HIV-1-Mediated Cell Fusion

A dye transfer assay was used for detection of HIV-1-mediated cell fusion as described (Jiang et al., 2000). For details, see the Supplemental Experimental Procedures.

Hemolysis

Hemolysis was determined as described (Helmerhorst et al., 1999). For details, see the Supplemental Experimental Procedures.

Structural Analysis

The methods used to determine the solution structures of the FP1-23 and VIR-165 peptides and of the complex are described in the Supplemental Experimental Procedures.

Biacore Analysis

Surface plasmon resonance studies are described in the Supplemental Experimental Procedures.

HIV-1 gp41 FP Random Mutants

Site-specific random mutagenesis of the HIV-1 NL4-3 molecular clone was performed by splice-overlap extension PCR using wobble primers. For details, see the Supplemental Experimental Procedures.

Statistical Analysis

IC_{50} values were compared using a two-tailed Student's *t* test. The PRISM package version 4.0 (Abacus Concepts, Berkeley, CA) was used for all calculations.

Supplemental Data

Supplemental data include Experimental Procedures, nine figures, and six tables and can be found with this article online at http://www.cell.com/cgi/content/full/129/2/263/DC1/.

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Accession Numbers

The structure of the VIR-165/FP complex has been submitted to the Protein Data Bank and Biological Magnetic Resonance Data Bank databases and has been assigned the accession numbers 2jnr and 15124, respectively.